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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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JUL 11 2002

ATTY.'S DOCKET: VESPER1

In re Application of:

Art Unit: 1645

TECH CENTER 1600/2900

S.J. VESPER

Examiner: SHAHNAN-SHAH

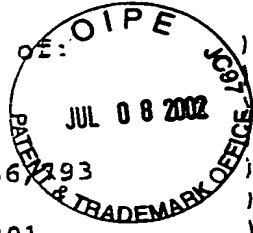
Serial No.: 09/866,193

Confirmation No. 5682

Filed: May 30, 2001

Washington D.C.

For: METHODS FOR ISOLATING  
AND USING FUNGAL  
HEMOLYSINS



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DECLARATION OF STEPHEN VESPER

I, Stephen Vesper, do declare that I am an inventor of the above-identified application. In order to demonstrate that extrapolating information in the specification as filed for *Stachybotrys chartarum* to other fungi, experiments were conducted under my direction and control to demonstrate that it is easy to determine which fungi produce hemolysin, and that it is easy to obtain hemolysin from a variety of fungi.

As demonstrated by the results shown in Table 1, one skilled in the art can readily determine if a fungus exhibits hemolytic activity.

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Table 1. Observations of hemolytic activity by indoor fungi. In each case, 10 µl of each conidial suspension was plated on sheep's blood agar and incubated at 37 and 23°C. The "asterisks" represent amount of growth. One asterisk is small amount of growth, two is moderate growth and three represents good growth. If there is no growth then there is no hemolysin. To ensure that the conidia were alive they were all grown at 23°C on SBA. (Only in a few cases were hemolysins produced at 23°C.)

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Fungal species	EPA #	Growth Hemolysin at		Growth Hemolysin at	
		37°C	37°C	23°C	23°C
<i>Absidia corymbifera</i>	133	+++	No	+++	No
<i>Acremonium strictum</i>	547	No	-	**	No
<i>Alternaria alternata</i>	628	*	No	**	No
<i>Aspergillus auricomus</i>	332	No	-	**	No
<i>Aspergillus caespitosus</i>	371	*	No	+++	No
<i>Aspergillus versicolor</i>	524	*	Yes	*	No
<i>Aspergillus candidus</i>	329	No	-	+	No
<i>Aspergillus carbonarius</i>	343	+	No	**	No
<i>Aspergillus cervinus</i>	351	No	-	+	No
<i>Aspergillus clavatus</i>	350	-	No	**	No
<i>Aspergillus flavipes</i>	610	*	Yes	**	No
<i>Aspergillus flavus</i>	532	+++	Yes	**	No
<i>Aspergillus fumigatus</i>	526	+++	Yes	**	No
<i>Aspergillus niveus</i>	361	**	Yes	**	No
<i>Aspergillus niger</i>	88	+++	Yes	+++	Yes
<i>Aspergillus ochraceus</i>	426	*	Yes	**	No
<i>Aspergillus paradoxus</i>	373	No	-	**	No
<i>Aspergillus parasiticus</i>	525	+++	Yes	**	No
<i>Aspergillus puniceus</i>	368	No	-	**	No
<i>Aspergillus restrictus</i>	458	No	-	*	No
<i>Aspergillus sclerotiorum</i>	237	-	Yes	**	No
<i>Aspergillus sydowii</i>	421	**	Yes	**	No
<i>Aspergillus tamarii</i>	607	+++	No	+++	No
<i>Aspergillus terreus</i>	231	+++	Yes	**	No
<i>Aspergillus unguis</i>	364	**	Yes	**	No
<i>Aspergillus ustus</i>	427	*	No	**	No
<i>Aspergillus versicolor</i>	524	*	Yes	*	No
<i>Aspergillus wentii</i>	608	No	-	**	Yes
<i>Aureobasidium pullulans</i>	701	No	-	-	No
<i>Chaetomium globosum</i>	396	**	No	+++	No
<i>Cladosporium cladosporioides</i>	178	No	-	*	No
<i>Cladosporium cladosporioides</i>	174	No	-	*	No
<i>Cladosporium herbarum</i>	69	No	-	*	No
<i>Cladosporium sphaerospermum</i>	416	No	-	*	No
<i>Emericella nidulans</i>	527	+++	Yes	**	Yes
<i>Emericella variegata</i>	684	*	Yes	**	No

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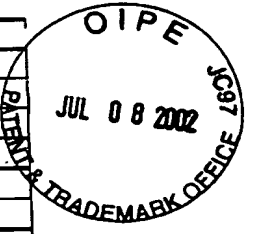
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<i>Epicoccum nigrum</i>	335	No	-	**	No
<i>Eurotium chevalieri</i>	530	*	No	*	No
<i>Monnensia echinata</i>	394	*	Yes	**	No
<i>Myrothecium verrucaria</i>	115	***	Yes	**	No
<i>Mucor racemosus</i>	138	No	-	***	No
<i>Faecilomyces varioti</i>	75	**	Yes	*	No
<i>Penicillium aethiopicum</i>	310	*	Yes	**	No
<i>Penicillium atramentosum</i>	601	No	-	**	No
<i>Penicillium aurantiogriseum</i>	441	No	-	**	No
<i>Penicillium brevcompactum</i>	435	No	-	**	Yes
<i>Penicillium caescens</i>	437	*	Yes	*	No
<i>Penicillium chrysogenum</i>	467	*	Yes	***	No
<i>Penicillium citreum</i>	277	No	-	*	No
<i>Penicillium citrinum</i>	448	*	Yes	*	No
<i>Penicillium coprophilum</i>	600	No	-	**	No
<i>Penicillium corylophilum</i>	434	No	-	*	No
<i>Penicillium crustosum</i>	445	No	-	**	No
<i>Penicillium decumbens</i>	430	*	Yes	*	No
<i>Penicillium digitatum</i>	316	No	-	*	No
<i>Penicillium expansum</i>	54	No	-	**	No
<i>Penicillium fellutanum</i>	431	*	Yes	*	No
<i>Penicillium glandicola</i>	449	No	-	**	No
<i>Penicillium griseofulvum</i>	456	*	Yes	*	No
<i>Penicillium implicatum</i>	452	No	-	*	No
<i>Penicillium islandicum</i>	616	*	Yes	*	No
<i>Penicillium italicum</i>	59	No	-	*	No
<i>Penicillium janthinellum</i>	521	**	Yes	**	No
<i>Paecilomyces lilacinus</i>	548	*	Yes	**	No
<i>Penicillium lividum</i>	292	No	-	*	No
<i>Penicillium melinii</i>	451	No	-	*	No
<i>Penicillium miczynskii</i>	443	No	-	**	No
<i>Penicillium olsonii</i>	523	No	-	**	No
<i>Penicillium oxalicum</i>	497	***	Yes	**	No
<i>Penicillium purpurogenum</i>	306	*	No	*	No
<i>Penicillium raistrickii</i>	442	No	-	**	No
<i>Penicillium restrictum</i>	613	*	Yes	*	No
<i>Penicillium roquefortii</i>	312	No	-	**	No
<i>Penicillium sclerotiorum</i>	453	No	-	**	No
<i>Penicillium simplicissimum</i>	603	*	Yes	**	No
<i>Penicillium spinulosum</i>	447	No	-	**	No
<i>Penicillium variable</i>	322	No	-	*	No
<i>Penicillium verrucosum</i>	440	No	-	**	No
<i>Penicillium waksmanii</i>	432	No	-	**	No
<i>Rhizopus stolonifer</i>	58	No	-	***	No



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<i>Scopulariopsis brevicaulis</i>	408	**	Yes	**	No
<i>Scopulariopsis brumptii</i>	409	-	No	+	No
<i>Scopulariopsis chartarum</i>	176	No	-	*	No
<i>Stachybotrys chartarum</i>	388	**	Yes	**	No
<i>Trichoderma asperellum</i>	538	**	No	**	No
<i>Trichoderma harzianum</i>	147	-	No	***	No
<i>Trichoderma longibrachiatum</i>	519	***	Yes	***	No
<i>Trichoderma viride</i>	405	No	-	***	No
<i>Ulocladium atrum</i>	629	+	Yes	**	No
<i>Ulocladium botrytis</i>	630	-	Yes	**	No
<i>Ulocladium chartarum</i>	631	-	No	**	No
<i>Wallemia sebi</i>	419	No	-	-	No



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As shown in Table 1, the screening for the production of a hemolysin is simply done by placing conidia (spores) of a given fungus on 5% sheep's blood agar (SBA) (Becton Dickinson, Sparks, MD) and incubating the plates at 37°C. In a week, 32 fungi were screened for hemolysin production by looking for typical darkening then clearing of the red blood cells around the colony. This is simple, inexpensive and fast. Then all one has to do is take a fungus that produce an hemolysin and grow in TSB broth as the patent teaches. Then collect the supernatant. This is important because the homogenate (like used for asp-hemolysin) introduces a huge number of other fungal proteins whereas the supernatant is relatively free of most other fungal proteins and makes the whole purification much easier and more meaningful since the secreted form of the hemolysin is the active form. Then the hemolysin is purified using standard protein purification procedure described in the patent:

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Step 1- size fractionation centrifugation; Step 2- ion chromatography; Step 3- gel filtration. Obviously there is slight variations in buffers, salt concentration, gel matrix etc. but these are accepted variations that any protein biochemist expects.

To demonstrate how easy it is to isolate other fungal hemolysins using the specified directives in the patent application, I isolated the hemolysin from *Penicillium chrysogenum* using the same steps which are growth of culture in tryptic soy broth (TSB), size fractionation centrifugation of supernatant, ion chromatography, and gel filtration.

The indoor fungus *Penicillium chrysogenum* was grown on pieces of dry wall, as described (19) and the conidia recovered. Approximately  $1 \times 10^8$  conidia were added to 500 ml of TSB. The cultures were incubated at 23°C for 48h on an incubator shaker at 100 rpm. Then the cultures were transferred to an incubator shaker and culture for 72 h at 34°C. The fungal mass was then removed by filtering through a Whatman 541 filter paper in a Buchner funnel. The recovered filtrate was centrifuged in a Millipore Centricon plus 30 filter apparatus with a MW cut-off of 30-kDa (Millipore, Bedford, MA) following the manufacturer's instructions. The concentrate was then subjected to ion exchange

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chromatography. DEAE-cellulose (Sigma, St. Louis, MO) was hydrated in 20 mM Tris-HCl pH 8.0 for 1 hr and then poured into a column giving a final bed of 3 x 0.5 cm. Then 0.5 ml of the concentrate was introduced on the top of the column. The bed was eluted with 5 ml of the 20 mM Tris-HCl buffer and five drop fractions were collected throughout the elution and then 10  $\mu$ l of each fraction was plated on sheep's blood agar (SBA) (Becton Dickinson, Sparks, MD) and hemolysis noted.

Then carboxy methyl cellulose (Sigma, St. Louis, MO) was hydrated in 20 mM Tris-HCl pH 8.0 for 24 h and then poured into a column giving a final bed of 3 x 0.5 cm. Then the five active fractions from the DEAE-cellulose ion chromatography were introduced on the top of the column. The bed was eluted with 5 ml of the 20 mM Tris-HCl buffer and five drop fractions were collected throughout the elution and then 10  $\mu$ l of each fraction was plated on sheep's blood agar (SBA) (Becton Dickinson, Sparks, MD) and hemolysis noted.

The five hemolytically active fractions from the second ion exchange chromatography were then subjected to gel filtration using Sephadex G 200 (Pharmacia, Piscataway, NJ) hydrated for 72 h in the running buffer containing 0.2 M sodium azide and poured into a chromatography column to give a final bed 0.25 by

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24 cm. Five drop fractions were collected at 1.5 ml per h using a fraction collector (ISCO, Lincoln, NE). Then 10  $\mu$ l of each fraction was plated on SBA and incubated at 37°C and hemolysis noted at 24 h. The five hemolytically active fractions from the first gel filtration were then subjected to gel filtration using Sephadex G 100-50 (Sigma, St. Louis, MO) hydrated for 5 days in the running buffer containing 0.4 M sodium azide, 5 mM EDTA, and 1 mM PMSF and poured into a chromatography column to give a final bed 0.5 x 14 cm. Five drop fractions were collected at 1.5 ml per h using a fraction collector (ISCO, Lincoln, NE). Then 10  $\mu$ l of each fraction was plated on SBA and incubated at 37°C and hemolysis noted at 48 h. The five most hemolytically active fractions from this second gel filtration were combined and then desalted twice using the D-Salt™ Polyacrylamide 6000 desalting column (Pierce, Rockford, IL.). The final desalted solution was frozen at -80°C and lyophilized using a Spin Vac (Savant Instruments, Farmingdale, NY) resulting in a lyophilized pellet.

#### Electrophoresis analysis

Native protein electrophoresis and SDS-PAGE were performed using the Bio-Rad Laboratories Mini-Protean™ 3 Cell and precast 4-15% Tris-HCL gels (BioRad, Hercules, CA), as per the manufacturer's instructions.



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Gels were stained with silver using the Bio-Rad Silver Stain Plus™ kit by following the manufacturer's instructions (Bio-Rad, Hercules, CA).

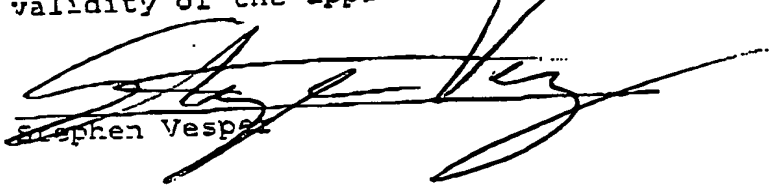
To determine if the chrysolysin was active, a purified preparation was divided in half and run in two separate wells in a precast 4-15% Tris-HCl gel. After native protein electrophoresis, half of the gel was stained with silver, as described above, and the other half was placed on an SEA plate and incubated at 37°C for 48 h and hemolysis process photographed.

Figure 1 shows the active purified hemolysin band from *P. chrysoygenum*. All of the steps described in the patent were used with modifications that any protein chemist would anticipate.

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 31 of the United States Code and that such willful false statements may jeopardize the

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validity of the application or any patent issued thereon:



~~Stephen Vespa~~

7-5-02

G:\bna\spalvesper\lpro\july 02 declaration

Figure 1. Purified hemoysin, chrysolysin, produced by *Penicillium chrysogenum*. Left panel shows purified band of chrysolysin in "native" gel. Center panel shows appearance of sheep's blood agar (SBA) after exposure to purified chrysolysin in "native" gel for 24 h and the right panel shows appearance of SBA after 48 h exposure (SBA incubated at 37°C).

